

Preliminary assessment of mating duration and prolonged post-copulatory associations in *Arrenurus* water mites (Actinotrichida: Parasitengonina: Hydrachnidia)

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Original research

ABSTRACT

Conflicts of interest between the sexes over control of mating can be reflected in various aspects of morphology and behaviour, including structure of genitalia and copulation duration. In *Arrenurus* water mites (Hydrachnidia: Arrenuridae) there are two main patterns of sperm transfer that differ in degree of potential male control of sperm uptake by females. In some species, males are equipped with an intromittent organ (the ‘petiole’) that is used to forcefully insert sperm into the female reproductive tract. In others, males lack an intromittent organ and females appear to push sperm into their reproductive opening themselves. Theory suggests that the amount of time spent in courtship after sperm transfer should differ between males with and without an intromittent structure. We predicted that male *Arrenurus* able to push sperm into the female’s reproductive tract (petiolate males) should spend less time courting females after transferring sperm than apetiolate males, which may have to ‘convince’ females to take up their sperm. Here, we examined durations of mating for 11 species of *Arrenurus* with males that differ in genital morphology: seven species with males equipped with a well-developed petiole (= ‘petiolate’ species) and four species with males that either completely lack a petiole or have a minute peg-like petiole that does not appear to function as an intromittent organ (= ‘apetiolate’ species). We tested whether males of petiolate species spend less time in the stage of courtship that takes place after sperm transfer (= ‘post-transfer courtship’) than apetiolate males. In contrast to our prediction, we found that species with well developed petioles spent significantly more time in post-transfer behaviours than species lacking petioles. The possible function of protracted post-transfer courtship in the genus *Arrenurus* is discussed.

Keywords sexual selection; sexual conflict; cryptic female choice; genitalia; reproductive morphology

Introduction

Sexual selection theory was initially proposed to explain the existence of sexual dimorphism in species in which there is little ecological differentiation between the sexes (Darwin 1859).

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The originally described scenarios relate to situations in which males compete for access to females, either through combat or through courtship displays in which females choose the winner (Darwin 1871). However, there are situations where males show morphological, physiological and behavioural adaptations that appear to bypass female choice. Sexual conflict theory argues that in some species, male genitalia and behaviour evolved to force females to mate (= coercion). If this reduces female fitness, this should select for female resistance to male coercive morphology and behaviour (Arnqvist and Rowe 2005). Evolutionary conflicts of interest can apply to which sex controls mating rate, copulation duration, female remating propensity and ultimately fertilization of eggs (Edvardsson and Canal 2006; Tong *et al.* 2021).

Similarly, although females may benefit from mating with additional 'higher quality' males after a first mating, it is in the first male's interest to prevent females from remating (Arnqvist and Rowe 2005; Firman *et al.* 2017). There are various adaptations to reduce polyandry including use of mating plugs by males, transferring manipulative seminal chemicals, and postcopulatory mate-guarding (Arnqvist and Rowe 2005; Firman *et al.* 2017). It has been experimentally shown for some species that prolonging the postcopulatory stage of mating increases probability of fertilization of eggs by sperm of a particular male, since more sperm and accessory ejaculate substances are moved into the female's reproductive tract over time (Eberhardt 1985; Arnqvist and Rowe 2005). In response to male adaptations to prolong mating duration, females have evolved counter-adaptations such as struggling in order to dislodge clinging males (Edvardsson and Canal 2006; Bergsten and Miller 2007).

Sperm-transfer behaviour in water mites of the genus *Arrenurus* Dugès, 1834 (Actinotrichida: Parasitengonina: Hydrachnidae) ranges from simple to very complex among around the ~1000 described species (Smit 2020; Więcek *et al.* 2021). In males, the hindbody (= cauda) may be simple and differ only slightly from the female's hindbody (Figure 1A), whereas in other species the males have elongated or complexly sculptured cauda. The most complex cauda are equipped with pygal lobes, various protrusions and an elaborate median intromittent organ (the petiole) (Figure 1B). The male's cauda and more anterior region of the hindbody have pairs of integumentary glands (= glandularia), some of which produce an adhesive secretion during mating that is used to glue the female to the male's hindbody (Jin *et al.* 1997). Moreover, males of some species have a spur-like extension on the fourth leg that is used to clasp the female's legs and hold her during the early stages of pairing (Jin *et al.* 1997; Proctor and Wilkinson 2001).

Among *Arrenurus* species there are two main modes of sperm transfer that appear to differ in the amount of control the female has over moving sperm into her genital tract (Figure 2). In one mode, males that lack an intromittent organ deposit spermatophores on the substrate and lower the female on top of the sperm packets on the spermatophores. The timing and mechanism of sperm uptake by the females of apetiolate species has not been definitively observed. In *Arrenurus globator* (O.F. Müller), clumps of sperm accumulate between the end of the male's cauda and the female's genital opening, so the female does not seem to have her genital flaps open at the time of spermatophore deposition (Böttger 1962). She may open them later while the male is engaged in post-transfer courtship, as suggested by Böttger (1962) for *A. globator*, or by grooming sperm into her genital opening after dismounting from the male, as Proctor (2003) suggested might be the case for *A. manubriator* Marshall. In the second mode, after depositing spermatophores, males gather the sperm on the tip of the petiole and push sperm into the female's genital tract with the petiole (e.g., *Arrenurus valdiviensis* K.O. Viets, Böttger 1965) (Figure 2D). Sexual conflict is likely more intense in species with males possessing well developed intromittent organs that appear to bypass female choice of sperm uptake, while female choice should be the stronger force of selection in species with males not equipped with such an organ (Proctor and Smith 1994; Proctor and Wilkinson 2001).

Both male and female *Arrenurus* of at least some species mate repeatedly in their lifespan, and therefore conflicts over mating rates and courtship duration may take place (Proctor and Wilkinson 2001; Proctor 2003). The duration of mating in different *Arrenurus* species seems to be largely controlled by males through various methods: the male clasping and manoeuvring

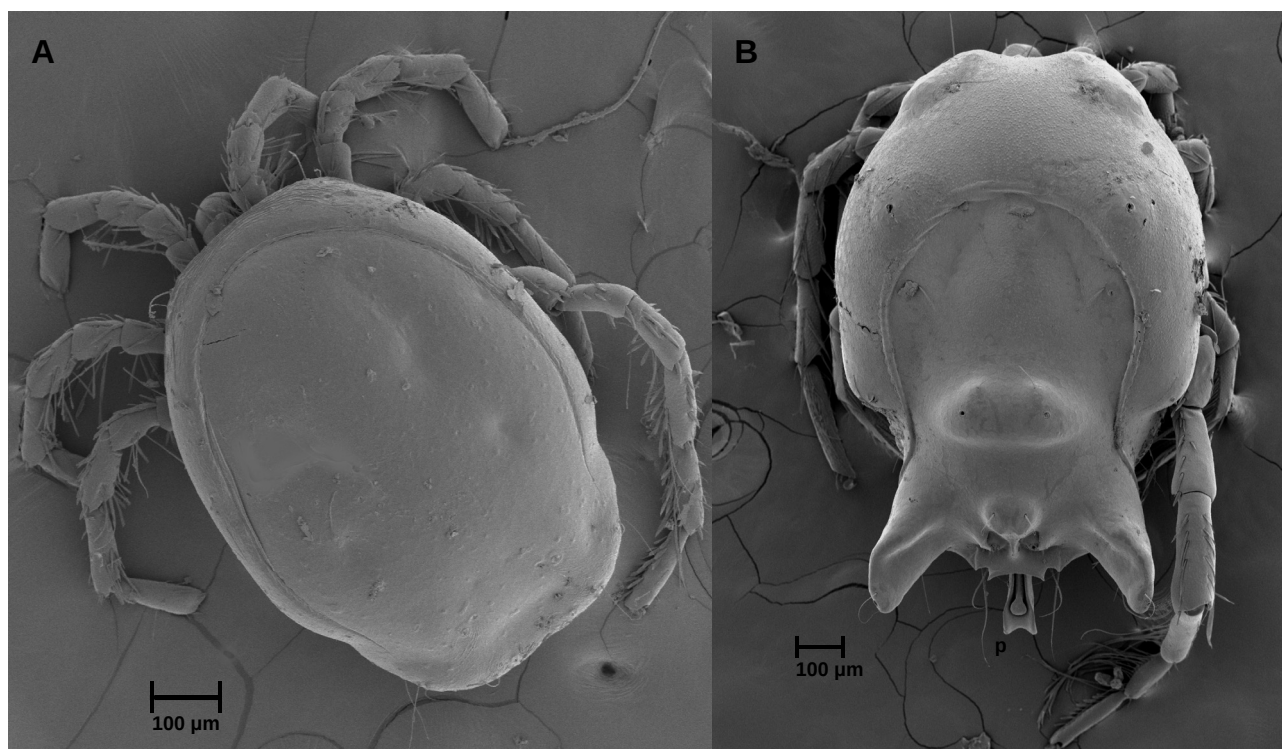


Figure 1 Scanning electron micrographs of males: A – apetiolate *Arrenurus fontinalis* Viets; B – petiolate *Arrenurus bicuspidator*. Abbreviation: p = petiole.

the female before and during coupling with the use of a spur-like extension on the fourth leg, gluing the female to his hindbody, and (in some species) using the petiole to insert sperm into the female. Separation of male and female at the end of the mating sequence also sometimes seems to be controlled at least in part by the male pushing the female in a way that breaks the binding of the glue (Proctor and Wilkinson 2001). Here, we observed full mating sequences and examined mating durations of *Arrenurus* species differing in the presence of a well-developed petiole in the male. We predicted that males lacking well-developed petioles should spend more time on post-deposition courtship in order to ‘encourage’ females to move sperm into their genital tracts. In contrast, we predicted that males equipped with petioles that forcefully push sperm into the female genital tract should spend less time on courtship following sperm transfer.

Materials and methods

Mite collection and maintenance

Arrenurus mites were collected by MW using a net with a mesh size of 250 µm in four locations in Poland: ponds at Adam Mickiewicz University (AMU) campus in Poznań (52°27'59.3"N 16°55'57.2"E), Bagnisko lake (53°29'55.7"N 16°28'42.3"E), Łagowo lake (52°19'33.6"N 15°17'16.8"E) and an unnamed pond near Zielona Góra (51°55'03.8"N 15°28'50.6"E), and one location in the Czech Republic in Miličovský pond (50°01'33.3"N 14°32'22.5"E). Live specimens were sorted by MW under a stereomicroscope in the laboratory.

Adult mites were separated by species and sex, and maintained in tissue culture plates with diameter 5 cm and 3 cm deep at ambient temperatures (~20-25 °C) in the laboratory. Deutonymphs collected in the field were maintained individually in their own well-plates (2

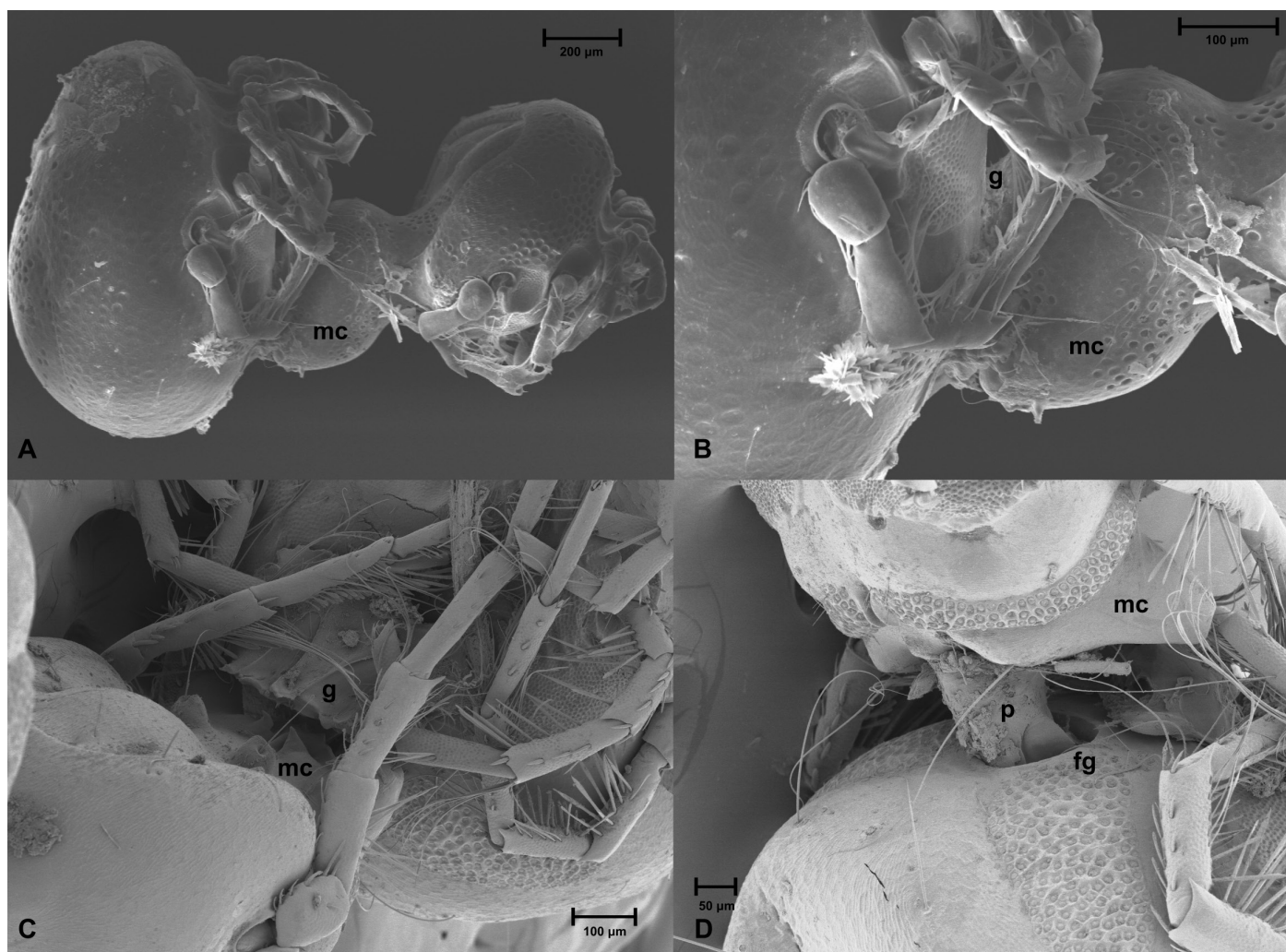


Figure 2 Scanning electron micrographs of *Arrenurus* spp. in copula: A, B – *Arrenurus globator* in mating position (lateral view): female glued to male's hindbody (no petiole); C, D – *Arrenurus* sp.: female glued to males dorsum, the petiole inserted in to female genital opening is shown. Abbreviations: fg = female's genital opening, g = adhesive glandular secretion gluing female to male's hindbody, mc = male's cauda (hindbody), p = petiole.

cm diameter, 1 cm deep; hereafter, culture well) until they transformed into adults and could be sexed. Both adults and deutonymphs were fed with ostracods, cladocerans and copepods from laboratory colonies established from material collected in local water bodies. Adult mites were identified to species based on morphology (Gerecke *et al.* 2016).

Mating observations

Female and male mites (whenever possible, virgin specimens) were held individually in culture wells filled with aged tap water for 24 h before observation. The culture wells had diameter of 2 cm and depth of 1 cm with bottom etched to provide more stable footing for the mites during mating.

We observed entire mating sequences for eight species of *Arrenurus*: six with petiolate males (*A. bicuspidator* Berlese, *A. bruzelii* Koenike, *A. claviger* Koenike, *A. cuspidator* (O.F. Müller), *A. cf. denticulatus* Motaş and *A. tricuspikator* (O.F. Müller)) and two with apetiolate males (*A. globator* and *A. stecki* Koenike). Details of mating behaviour of these species are in Więcek (2016). A culture well with a single female was placed under an Olympus DP71

digital camera with CellD 2.8 software (Olympus Soft Imaging Solutions GmbH) connected to Zeiss Stemi 2000-C stereomicroscope with a moveable light source. Video recording began when a male was introduced to a culture well with a conspecific female. Recording was terminated upon physical separation of individuals after mating. The mating sequences of *A. cf. denticulatus* and *A. globator* (three pairings) were not recorded on video, and observations described in Results are based on notes made in real time by MW. All observations took place at ambient temperatures (~20-25 °C).

We divided mating sequences into the following categories, listed in temporal order: (a) pre-pairing stage prior to the female being glued to the male, (b) behaviours from initial pairing to the end of spermatophore deposition and sperm transfer and (c) post-deposition behaviour. In all categories except pre-pairing, the female was attached to the male. For statistical analysis we calculated the following: (a) pre-pairing duration = total time from introduction of a male into a female's well to the female being glued onto the male; (b) duration of behaviours from initial pairing to the end of sperm transfer (deposition of spermatophores was assumed based on male 'dipping' movements); (c) post-deposition behaviour = time male and female were still attached to each other after spermatophore deposition/sperm transfer; (d) total duration of mating = total time that male and female were attached (b+c); Unlike in Lundblad (1929) and Więcek (2016), we treated the male behaviours of lateral waving and alternate bending of left and right legs as part of post-deposition behaviour.

SEM images of couples in copula were made in the Department of Earth and Atmospheric Sciences, University of Alberta. After dehydration through an alcohol-HMDS (hexamethyldisilazane) series, mites were mounted on stubs using double-sided tape, sputter coated with gold, and examined using a JEOL 630 I field emission scanning electron microscope (SEM). SEM photographs were edited in Photoshop 6.0.

Statistical analyses

Data were first tested for significant deviations from normal distribution with Shapiro-Wilk tests. The Fligner-Killeen test was applied to test for equal coefficient of variation in two samples. In each analysis, behavioural data did not fulfill at least one of the assumptions of a parametric test. Therefore, we applied non-parametric tests. Mann-Whitney U tests were used for comparing the two groups of species (apetiolate vs petiolate) for durations of particular stages of mating. A Kruskal-Wallis test was performed for each stage of mating, followed by Dunn's post hoc test in order to assess between which species pairs statistically significant differences are observed. For each stage of mating 55 Dunn's tests were run (results in Supplemental Tables S1-S4). The Bonferroni correction was not used here, as it is very conservative, and we view these results as a preliminary assessment for further investigation (Perneger 1998; Cabin and Mitchell 2000). $P < 0.05$ was used as the upper cutoff for significant differences. Data on differences in percentage of time spent on post-deposition behaviours were log transformed prior to analysis. For each species of *Arrenurus* we used 43 created box plots to show medians, 25-75 percent quartiles and ranges of minimal and maximal values. All analyses were run with the statistical software PAST 4.03 (Hammer *et al.* 2001).

In at least some species of *Arrenurus*, the duration of the pre-pairing stage of mating can be affected by female age and whether females had been previously exposed to males (HP, pers. obs.). Because mating status and age of mites were not standardized, we excluded pre-pairing duration from statistical tests and only present summary statistics. In order to obtain a larger sample size to test our predictions, the results were pooled with the data obtained by Proctor and Wilkinson (2001) for three North American species: petiolate *A. sp. nr. reflexus* Marshall and apetiolate *A. manubriator* and *A. rufopyriformis* Habeeb.

Results

In this study 60 pairings of eight *Arrenurus* species from Europe (*A. bicuspidator*, *A. bruzelii*, *A. claviger*, *A. cuspidator*, *A. cf. denticulatus*, *A. globator*, *A. stecki*, *A. tricuspidator*) and 3 species from Canada (*A. manubriator*, *A. sp. nr. reflexus*, *A. rufopyriformis*) were analysed (Table 1).

Box plots presenting durations of particular stages of mating are shown in Figures 3 and 4. Total mating duration ranged from 29.00 ± 5.00 minutes in apetiolate *A. stecki* to 630.00 ± 79.00 minutes in petiolate *A. bicuspidator* (Table 1). Total duration of mating in petiolate species was significantly longer (mean = 394.82 ± 22.02 min, N = 34 pairings) than in apetiolate species (mean = 94.04 ± 10.45 min, N = 26 pairings) (Mann-Whitney U Test, $P < 0.0001$). There was no significant difference in duration of behaviours from initial pairing to the end of spermatophore deposition and sperm transfer between petiolate (mean = 26.03 ± 2.48 min, N = 33 pairings) and apetiolate (mean = 46.11 ± 9.04 min, N = 26 pairings) species (Mann-Whitney U Test, $P = 0.33$). Petiolate species spent significantly more time on post-deposition behaviour (mean = 366.42 ± 22.08 min, N = 33 pairings) than apetiolate species (mean = 47.88 ± 6.67 min, N = 26 pairings) (Mann-Whitney U Test, $P < 0.0001$, both as measured in minutes and as a percentage of total mating time). Kruskal-Wallis tests showed overall significance ($P < 0.05$) in the differences between the examined species for the total duration of mating (Kruskal-Wallis H (10) = 54.23, $P < 0.0001$), duration of behaviours from initial pairing to the end of spermatophore deposition and sperm transfer (Kruskal-Wallis H (10) = 42.21, $P < 0.0001$) and duration of post-deposition behaviours (Kruskal-Wallis H (10) = 49.53, $P < 0.0001$). Post-hoc tests did not confirm significant differences (Dunn's test, $P < 0.05$) for most apetiolate-petiolate species pairs for the duration of behaviours from initial pairing to the end of spermatophore deposition and sperm transfer (Table S2). In contrast, significant differences were found between apetiolate-petiolate species pairs for the total duration of mating (except for *A. globator* - *A. bruzelii* ($P = 0.062$), *A. manubriator* - *A. bruzelii* ($P = 0.085$) and *A. manubriator* - *A. sp. nr. reflexus* ($P = 0.053$)) (Table S1). Moreover, clear differences throughout apetiolate-petiolate species pairs were found for duration of post-deposition behaviours with only a few exceptions: *A. globator* -

Table 1 Mean time ($\pm$ SE) spent on particular stages of mating in different species of the genus *Arrenurus*. The total duration of mating does not include duration of the pre-pairing stage. Post-deposition behaviour is included in total duration of mating and is expressed both in minutes and as percentage of the total time spent on mating.

Table1

Species	N	Pre-pairing duration (min $\pm$ SE)	Total duration of mating (min $\pm$ SE)	Post-deposition behaviour min $\pm$ SE	% $\pm$ SE
petiolate group					
<i>A. sp. nr. reflexus</i> ¹	10	8.06 ± 3.85	318.3 ± 22.62	293.2 ± 19.54	92.41 ± 0.9
<i>A. cuspidator</i>	3	43.33 ± 28.87	598.00 ± 79.37	553.33 ± 74.37	92.47 ± 1.65
<i>A. bicuspidator</i>	2	35.00 ± 5.00	630.00 ± 79.00	610.5 ± 76.5	95.9 ± 1.0
<i>A. tricuspidator</i>	3	18.33 ± 6.0	$422.67 \pm 41.99^*$	$423 \pm 70.0^*$	$96.15 \pm 1.25^*$
<i>A. bruzelii</i>	9	29.6 ± 12.37	303.55 ± 20.42	272.78 ± 17.15	89.95 ± 1.23
<i>A. claviger</i>	4	18.00 ± 4.24	438.00 ± 10.92	405.25 ± 19.72	92.37 ± 2.42
<i>A. cf. denticulatus</i>	3	29.33 ± 5.36	478.33 ± 34.06	452.33 ± 38.64	94.50 ± 1.55
mean for petiolate	34	25.95 ± 4.50	455.55 ± 47.45	430.05 ± 46.90	93.39 ± 0.84
apetiolate group					
<i>A. globator</i>	7	84.00 ± 16.00	123.57 ± 20.62	87.43 ± 15.50	70.35 ± 1.50
<i>A. stecki</i>	2	32.00 ± 22.00	29.00 ± 5.00	16 ± 7.00	52.55 ± 15.05
<i>A. manubriator</i> ¹	7	N/A	132.57 ± 18.47	25.57 ± 3.28	21.21 ± 3.86
<i>A. rufopyriformis</i> ¹	10	3.2 ± 0.94	59.4 ± 4.4	42.2 ± 4.03	70.21 ± 1.46
mean for apetiolate	26	39.73 ± 23.64	86.13 ± 25.06	42.8 ± 15.83	53.58 ± 11.57

¹ data from Proctor and Wilkinson (2001)

* calculated from two of the three pairings because of the difficulties with interpretation of displayed behaviours

A. tricuspidator ($P = 0.055$), *A. globator* - *A. bruzelii* ($P = 0.14$) and *A. globator* - *A. sp. nr. reflexus* ($P = 0.086$) (Table S3). For post-deposition behaviour expressed as percentage of the total time spent on mating all comparisons between apetiolate-petiolate species pairs were statistically significant (Dunn's test, $P < 0.05$) (Table S4).

Discussion

We had expected that *Arrenurus* males with a well-developed intromittent structure (the petiole) would spend less time in courtship behaviours after sperm transfer than apetiolate males, which appear to have less coercive control over whether sperm moves into the female's reproductive tract. Contrary to our prediction, males of petiolate species spent more time in post-deposition behaviours than males of apetiolate species, both absolutely (mean number of minutes) and relatively (as percentage of total time spent mating) (Table 1). Although both petiolate and apetiolate species exhibited prolonged post-deposition associations, in petiolate species post-deposition behaviour made up $> 90\%$ of total mating time in contrast to $\sim 50\%$ in apetiolate species. However, there was a considerable variation in time spent on post-transfer behaviours among apetiolate species (Table 1).

Why might males of both petiolate and apetiolate species show such long periods of attachment to females after transferring sperm? Protracted courtship after sperm transfer, or simply lengthy male attendance, occurs in many animal groups and is thought to reduce the probability of the female being inseminated by a subsequent male (mate guarding, Arnqvist and Rowe 2005). For example, Radwan and Siva-Jothy (1996) demonstrated that males of the terrestrial mite *Rhizoglyphus robini* Claparède (Acaridae) increase fertilization of eggs by their own sperm by prolonging attachment to a female and preventing other males from mating with her. Male courtship and postmating behaviours may also encourage females to preferentially use sperm of a particular male and thus can be under selection by cryptic female choice (Firman *et al.* 2017). Conversely, in this stage of mating males can chemically manipulate a female's physiology by stimulating oviposition or decreasing production of sex pheromones attracting males (Arnqvist and Rowe 2005; Mazzi *et al.* 2009). We hypothesize that this could be an explanation of the functions of both the periods of motionlessness and the courtship behaviour after sperm transfer in the examined species of *Arrenurus*.

Why might petiolate males spend significantly more time in post-deposition behaviours than apetiolate ones? Males of apetiolate and petiolate species may differ in the amount of sperm competition they face, if, in addition to pushing sperm into the female's genital opening, the petiole also acts to remove or displace sperm deposited by previous males as do, e.g., the genitalia of male damselflies (Waage 1979). If so, the greater duration of post-transfer behaviour in petiolate species may reflect the lengths to which such males must go to increase the likelihood that females will use their sperm and not that of a previous or subsequent mate. To test this idea, the paternity of egg clutches could be assessed in matings in which the second male is allowed to complete the entire mating sequence or is interrupted at various times after sperm transfer.

Finally, although optimal copulation duration can be a subject of conflict between the sexes, which may be the case in the examined *Arrenurus*, it has been observed that females can sometimes benefit from prolonged copulations, e.g., if they receive larger ejaculates that can be used in part for nutrition (e.g. bruchid beetles, Edvardsson and Canal 2006). Therefore, further research should be done to determine if prolonged post-copulatory associations in *Arrenurus* mites influence female's lifetime fecundity as well as male's paternity.

The strength of our statistical interpretation of differences in mating durations was limited by sample size. Laboratory observations of mating behaviour in *Arrenurus* mites are affected by several factors. First, most *Arrenurus* species have a complex life cycle with larvae that parasitize insect hosts, which makes establishing laboratory colonies of mites challenging except for some rare cases of species that forego larval parasitism such as *A. manubriator* (see

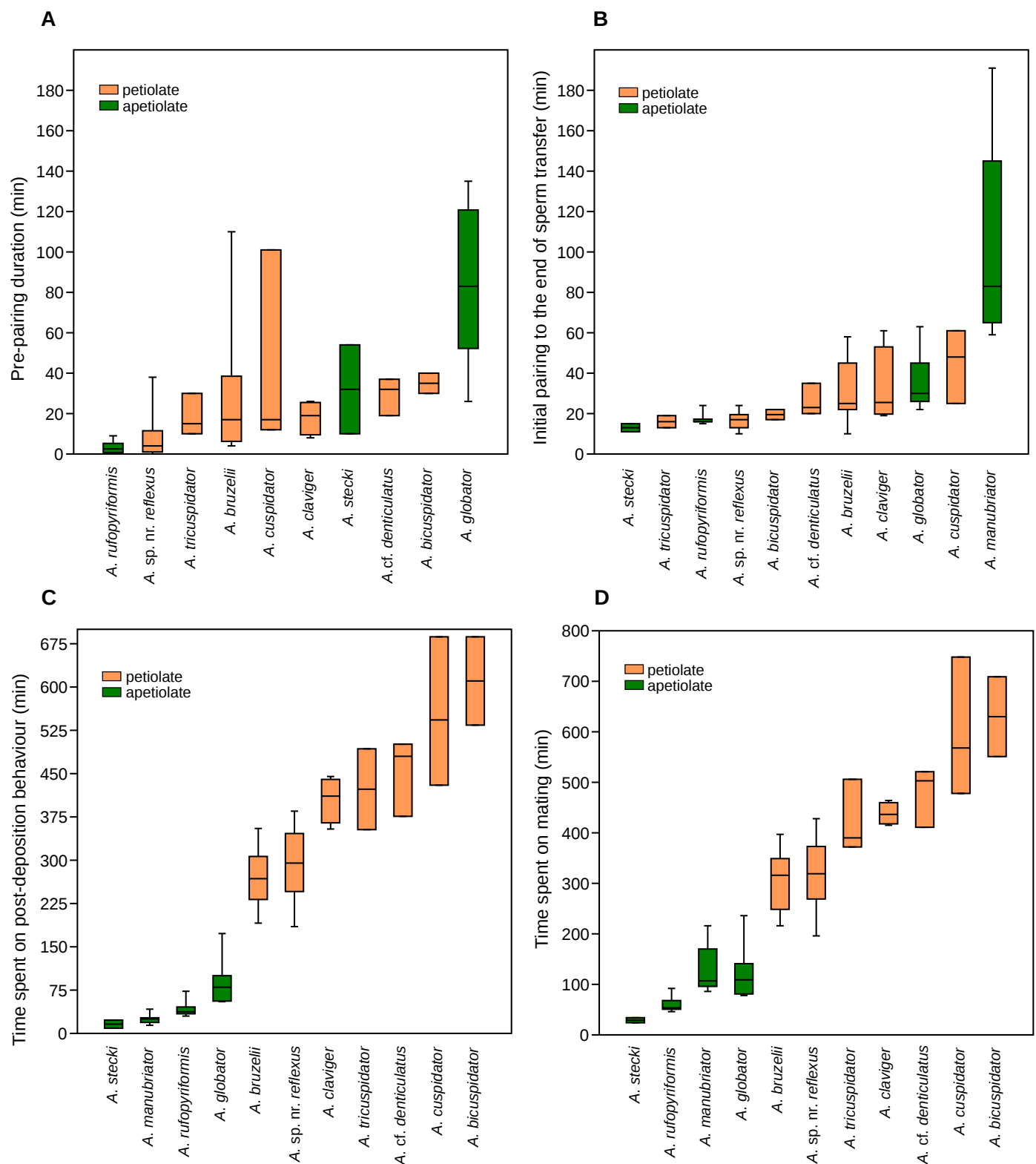


Figure 3 Durations of different stages of mating (in min): A – pre-pairing stage; B – behaviours from initial pairing to the end of spermatophore deposition; C – post-deposition behaviour; D – total time spent on mating. The 25-75 percent quartiles are drawn using a box. The medians are shown with a horizontal line inside the box. The minimal and maximal values are visualized with horizontal lines (“whiskers”). Petiolate species are indicated by light orange, and apetiolate ones by dark green.

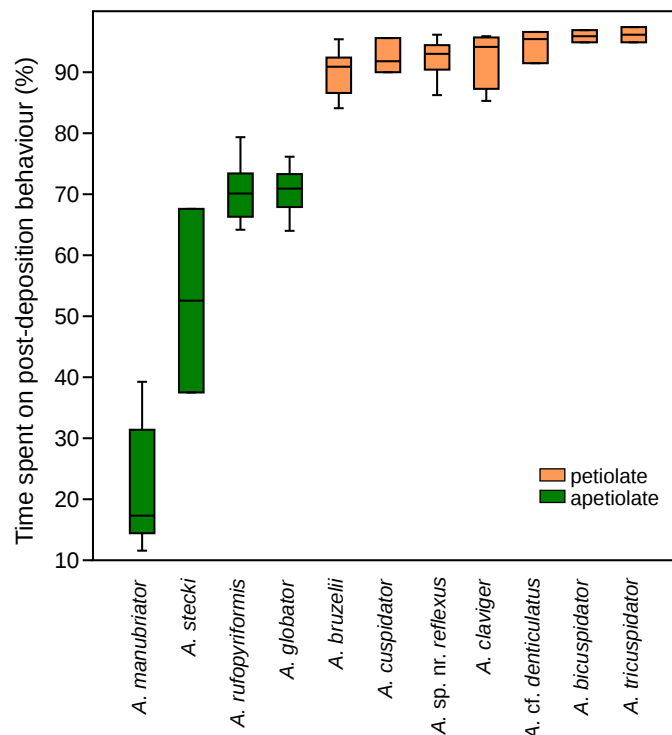


Figure 4 Time spent on post-deposition behaviour (in %). Post-deposition behaviour is expressed as percentage of the total time spent on mating. The 25-75 percent quartiles are presented using a box. The medians are shown with a horizontal line inside the box. The minimal and maximal values are presented with the “whiskers”. Petiolate species are indicated by light orange, and apetiolate ones by dark green.

Smith and Hagman 2002). Second, field-collected deutonymphs raised to adults in captivity do not always show interest in mating (MW, pers. obs.), which may be caused by inappropriate food offered (prey species of water mites in general are largely unknown, Vasquez *et al.* 2021). Third, particular species collected in the field engage in mating in different periods of the year, in some cases only through a few weeks in season (MW, pers. obs). Finally, some species from springs and streams may not show interest in mating in standing water in laboratory conditions.

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Conflict of interest

The authors have no conflict of interest to declare.

Data statement

Raw data are available from the corresponding author upon request, with the provision that this article will be cited in any publication using the raw data.

Preprint version

A preprint version has been deposited in bioRxiv <https://www.biorxiv.org/content/10.1101/2021.05.23.445345v1>

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